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Biochemical Studies on the Metabolic Activation of Halogenated Alkanes

by Kevin H. Cheeseman,* Emanuele F. Albano,† Aldo Tomasi,‡ and Trevor F. Slater*

This paper reviews recent investigations by Slater and colleagues into the metabolic activation of halogenated alkanes in general and carbon tetrachloride in particular. It is becoming increasingly accepted that free radical intermediates are involved in the toxicity of many such compounds through mechanisms including lipid peroxidation, covalent binding, and cofactor depletion. Here we describe the experimental approaches that are used to establish that halogenated alkanes are metabolized in animal tissues to reactive free radicals. Electron spin resonance spectroscopy is used to identify free-radical products, often using spin-trapping compounds. The generation of specific free radicals by radiolytic methods is useful in the determination of the precise reactivity of radical intermediates postulated to be injurious to the cell. The enzymic mechanism of the production of such free radicals and their subsequent reactions with biological molecules is studied with specific metabolic inhibitors and free-radical scavengers. These combined techniques provide considerable insight into the process of metabolic activation of halogenated compounds. It is readily apparent, for instance, that the local oxygen concentration at the site of activation is of crucial importance to the subsequent reactions; the formation of peroxy radical derivatives from the primary free-radical product is shown to be of great significance in relation to carbon tetrachloride and may be of general importance. However, while these studies have provided much information on the biochemical mechanisms of halogenated alkane toxicity, it is clear that many problems remain to be solved.

Introduction

The purpose of this paper is to review the investigations carried out by this group (principally at Brunel University) over recent years into the role of free radicals in the toxicity of halogenated alkanes. Carbon tetrachloride (CCl_4) is the prime example of an hepatotoxic haloalkane, and the biochemical mechanisms of its toxic effects have been intensively investigated over many years. While CCl_4 is no longer a clinically important hepatotoxin, it still has immense value as an experimental model agent. The methodology developed for the study of CCl_4 activation is now finding extensive application in the study of the toxicity of other haloalkanes and many other xenobiotics. That situation is reflected in this paper where it will be evident that although our principal experience has been in using CCl_4 we have a growing interest in other toxic haloalkanes. Our presentation can be broadly divided into three main sections: the demonstration of the formation of free radical metabolites from haloalkanes, the measurement of the reactivity of such radicals, and the study of the enzymatic mechanisms of haloalkane activation. While such a division is somewhat arbitrary, it is a convenient basis for the following review.

*Department of Biochemistry, Brunel University, Uxbridge, UB8 3PH, UK.
†Istituto di Patologia Generale, Corso Raffaello 30, 10125 Torino, Italy.
‡Istituto di Patologia Generale, Via Campi 287, 41100 Modena, Italy.

Studies on the Pathways of CCl_4 Metabolism: A Synopsis

Although the main histopathological features of CCl_4-induced liver injury had been fully described by 1936 (*1*), the biochemical mechanisms by which CCl_4 exerts its hepatotoxic actions were not investigated in detail until the 1960s, and have not been fully clarified to the present day. Prior to the 1960s the predominant view was that CCl_4 might act on the liver by a simple solvent action; early studies on the hepatoxicity of CCl_4 are fully reviewed by Recknagel (*2*). It is now a fundamental tenet that CCl_4 has to undergo metabolic activation in order to exert its full range of toxic effects.

In 1966, two papers appeared, virtually simultaneously, presenting similar theories for the mechanism of the hepatotoxicity of CCl_4 (*3,4*). Each proposed that the mechanism depended on the metabolism of CCl_4 to a free-radical product capable of initiating lipid peroxidation. Emphasis was placed on the importance of lipid peroxidation as a damaging reaction of potentially great

significance in cytotoxicity. Slater postulated that the free-radical product was the trichloromethyl free radical ($CCl_3^{\bullet}$) and that it was formed by interaction with endogenous radicals, perhaps involved in an enzymic process in the endoplasmic reticulum. In putting forward their proposals, Slater and Recknagel had to show why a theory based on the metabolic activation of CCl_4 accounted for those characteristic features of CCl_4 hepatotoxicity that could not be explained by the simple lipid-solvent hypothesis. The principal features of the concept of metabolic activation had previously been proposed by Miller and Miller (*5*) for chemical carcinogens, but the application of the concept to CCl_4 required a new insight into experimental data that had already been published. Those data included the following major contributions:

In 1951, McCollister et al. (*6*) had demonstrated that CCl_4 is metabolized *in vivo* to products including chloroform, CO_2, and urea. Butler (*7*), in perhaps the most provocative of these early papers, confirmed CCl_4 metabolism *in vivo* and proposed that CCl_4 toxicity depended on the homolytic fission of the C-Cl bond to radical products. Reynolds (*8*) reported that radiolabeled CCl_4 administered to rats became covalently bound to liver protein. Rubinstein and colleagues (*9,10*) demonstrated the role of the endoplasmic reticulum in the metabolism of CCl_4 to $CHCl_3$ and CO_2 *in vitro*. Wirtschafter and Cronyn (*11*) proposed that CCl_4 was converted to a radical form by interaction with endogenous free radicals. It was shown that CCl_4 stimulated lipid peroxidation in liver homogenates (*12*) and in microsomes plus cytosol (*13*); both of these groups recognized that the cytosolic fraction was required.

The proponents of the activation theory for CCl_4 (*3,4*) drew these threads together and added more experimental evidence in support of their proposals. Slater suggested that the relative toxicity of the halogenated methanes depended on their respective bond dissociation energies that would dictate the ease of homolytic free-radical formation; this, for instance, would explain the high toxicity of $CBrCl_3$ in which the C-Br bond is weaker than a C-Cl bond in CCl_4.

The activation theory was strengthened and essentially completed by papers appearing shortly thereafter. McLean and McLean (*14*) established the role of the microsomal drug-metabolizing enzymes in determining the toxicity of CCl_4 and proposed that these enzymes were involved in the metabolism of CCl_4. Gregory (*15*) proposed that activation of CCl_4 proceeded by a mechanism of electron capture, rather than homolysis and this scheme, in which $Cl^{\bullet}$ radicals are not produced, is generally accepted:

$$CCl_4 \xrightarrow{e^-} CCl_3^{\bullet} + Cl^-$$

Slater (*16*) demonstrated that CCl_4-dependent microsomal lipid peroxidation required a source of NADPH. Fowler (*17*) reported hexachloroethane (C_2Cl_6) as a metabolite of CCl_4 *in vivo*, a result consistent with the formation and dimerization of $CCl_3^{\bullet}$ radicals. The basis for the activation theory was thereby established.

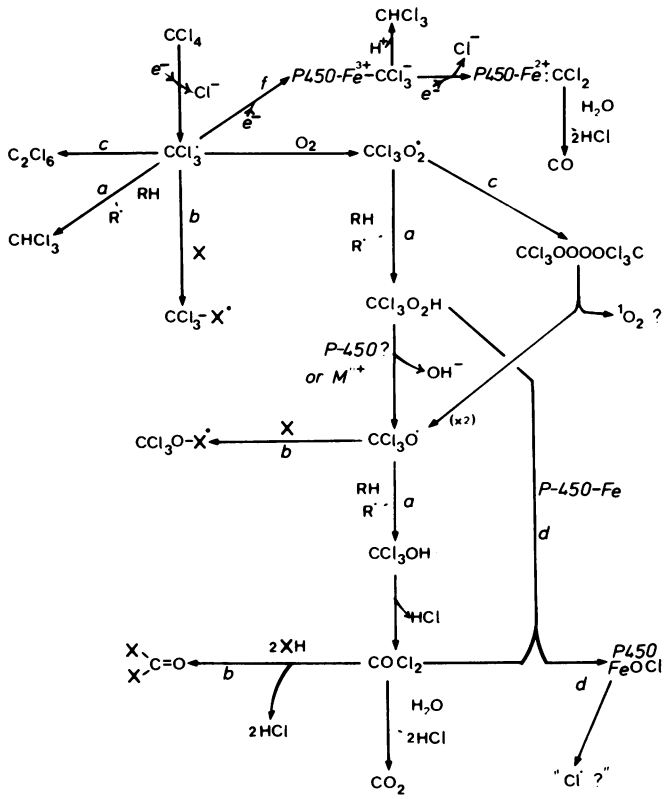

FIGURE 1. Pathways of metabolism of CCl_4. Following activation to $CCl_3^{\bullet}$, subsequent steps are highly dependent on the local oxygen concentration. Steps marked (a) show hydrogen abstraction; if RH is a polyunsaturated fatty acid then lipid peroxidation may ensue. Steps marked (b) show covalent binding to biomolecules (represented by X). Steps marked (c) show radical dimerization; in the case of $CCl_3O_2^{\bullet}$ this is speculative for the biological situation (*113*). Step (d) shows formation of electrophilic chlorine (represented as "Cl") as demonstrated by Pohl et al. (*23,24*). Step (f) is an anaerobic pathway via a carbene intermediate and is probably of minor significance (*29,30*).

Since then the metabolism of CCl_4 has been investigated intensively. The metabolites of this simple compound are manifold, and the pathways by which they arise are not yet fully understood. This situation is undoubtedly due to the initial product of CCl_4 activation being a relatively reactive free radical. The subsequent reactions of this intermediate can be conveniently divided into aerobic and anaerobic pathways (see Fig. 1).

Aerobic Metabolism

The aerobic pathway of CCl_4 metabolism terminates in CO_2, a product that was first detected by McCollister et al. (*6*). Seawright and McLean (*18*) studied the metabolism of CCl_4 to CO_2 in rat liver microsomes and showed that it required NADPH and was blocked by the P-450 inhibitor SKF 525A and the radical scavenger promethazine. It seemed likely that phosgene ($COCl_2$)

was the precursor of CCl_4-derived CO_2. More recently, Shah et al. (*19*) demonstrated that CCl_4 is metabolized to $COCl_2$ by rat liver homogenate. Kubic and Anders (*20*) showed that this was a microsomal process and that the oxygen atom was derived from molecular oxygen, which they took to indicate the involvement of a P-450-catalyzed oxygenase reaction. Using a model heme system of CCl_4 metabolism, Mansuy (*21*) considered that $COCl_2$ could arise from interaction of O_2 and a P-450-carbene or P-450-carbanion complex, or simply by the reaction of O_2 with free $CCl_3^{\bullet}$. In fact, O_2 reacts extremely rapidly with $CCl_3^{\bullet}$ to yield the trichloromethylperoxy free radical ($CCl_3O_2^{\bullet}$) as discussed later, and it is thus unnecessary to postulate P-450-mediated oxygenation in the usual sense. The rapid reaction of $CCl_3^{\bullet}$ with O_2 was measured in this department by Packer and it was suggested at that time that $COCl_2$ and CO_2 might arise via the formation of the $CCl_3O_2^{\bullet}$ radical (*22*). This hypothesis has latterly been taken up by Pohl and colleagues, who have further studied the aerobic metabolism of CCl_4 through to $COCl_2$ and have reported another product, namely "electrophilic chlorine" (*23,24*). This currently unidentified product can be considered analogous to $Cl^{\bullet}$ and may represent a further potentially toxic metabolite of CCl_4.

However, the significance of $COCl_2$ formation in CCl_4 hepatoxicity is not yet clear. In comparison with $CHCl_3$, CCl_4 produces relatively small amounts of $COCl_2$. *In vivo*, $CHCl_3$ administration reduces hepatic GSH considerably but CCl_4 administration reduces hepatic GSH only slightly, if at all (*25,26*). Accordingly, diglutathionyl dithiocarbonate, the product of the reaction of $COCl_2$ and GSH, is found in the bile of $CHCl_3$-treated rats at 25 times the level of that found in CCl_4-treated rats and its formation by microsomes *in vitro* with these two substrates is in the same proportion (*26*). These relatively large differences in the production of $COCl_2$ in livers exposed to $CHCl_3$ or CCl_4 should be contrasted with the much higher hepatotoxic activity of CCl_4 compared with $CHCl_3$. These routes of CCl_4 metabolism are shown in Figure 1. The tetroxide intermediate shown in Figure 1 is hypothetical, and it is not clear if dimerization of $CCl_3O_2^{\bullet}$ radicals is likely under physiological conditions. Similarly, the alkoxy radical derivative ($CCl_3O^{\bullet}$) seems a likely intermediate but has not yet been demonstrated.

Anaerobic Metabolism

It has been clearly established (*27*) that CCl_4 is metabolically activated under anaerobic conditions to give a much higher yield of covalently bound product than is found under corresponding aerobic conditions. This aspect of the metabolism of CCl_4 is discussed in detail later. Wolf et al. (*28*) showed that under anaerobic conditions a small amount of CO is produced from CCl_4 by liver microsomes, and they postulated that the precursor of this was the dichlorocarbene-P-450 ligand, itself the product of a carbanion intermediate (see Fig. 1). In this case the oxygen atom of CO comes from a water molecule. The significance of carbene formation was further investigated by Ahr et al. (*29*) who concluded that the products of anaerobic metabolism (other than CO) were principally derived from $CCl_3^{\bullet}$ and that the carbene intermediates were probably of little physiological significance. Kubic and Anders (*30*) showed that $CHCl_3$ formation was normally due to hydrogen abstraction by $CCl_3^{\bullet}$ rather than protonation of the carbanion intermediate, as judged by incubating liver microsomes in D_2O and measuring $CDCl_3$ formation. The latter route, however, appeared to be of more significance in phenobarbitone-treated rats.

It is clear that CCl_4 metabolism can proceed through aerobic and anaerobic routes and that the key initial step is the production of the $CCl_3^{\bullet}$ free radical. It follows that the next major controlling factor is the local oxygen concentration that will determine the relative flux through each pathway. Thus, under anaerobic conditions covalent binding and $CHCl_3$ production are favored; under aerobic conditions these will be reduced in favor of $COCl_2$ and CO_2 production. In normal aerobic microsomal incubations products of both pathways are found; probably the local O_2 concentration of the microenvironment of the site of activation is critical in determining certain features of the injurious reactions produced by CCl_4.

Demonstration of the Formation of $CCl_3^{\bullet}$ from CCl_4

By the early 1970s it had become generally accepted that CCl_4 undergoes a metabolic activation in the endoplasmic reticulum. However, there was no direct evidence for the formation of the $CCl_3^{\bullet}$ radical that was postulated to be the primary metabolite. Indirect evidence for its formation had been obtained; however, the formation of C_2Cl_6 as a product of CCl_4 metabolism (*17*) is most probably due to the dimerization of $CCl_3^{\bullet}$ radicals. Moreover, double-label radioisotope experiments measuring covalent binding of [^{14}C]- and [^{36}Cl]-CCl_4 were consistent with the binding of the $CCl_3^{\bullet}$ radical (*31,32*).

The method of choice for the detection of free-radical species is electron spin resonance (ESR) spectroscopy. However, attempts to detect the $CCl_3^{\bullet}$ free radical in whole liver or in liver fractions exposed to CCl_4 using direct ESR analysis were not successful (*33–36*). This is most probably due to the low steady-state concentration of this radical species in biological systems: its rate of formation may be rather low and its chemical reactivity is relatively high (*37*). The steady-state concentration of $CCl_3^{\bullet}$ is therefore probably below the detection limit of ESR spectroscopy (ca. 10^{-6} M). For this reason, the technique of spin trapping (*38*) has been used to demonstrate unequivocally the formation of the $CCl_3^{\bullet}$ radical. This technique utilizes a spin trap that does not by itself give rise to an ESR signal, but which reacts with a free radical to yield a relatively stable free-radical adduct that progressively accumulates to concentra-

tions readily detectable by ESR spectroscopy. Spin traps generally possess either a nitrone or a nitroso functional group and form nitroxyl radical adducts. The spin traps that have been most commonly used in biological systems in the period 1975–1982 are 2-methyl-2-nitrosopropane (MNP), phenyl-*N*-*tert*-butyl nitrone (PBN), 4-pyridyl-*N*-oxide-*tert*-butyl nitrone (POBN), and 5,5-dimethylpyrroline-*N*-oxide (DMPO); these are shown in Figure 2. Perhaps the most difficult aspect of this technique is the correct assignment of the nitroxide radical spectrum to the original radical species. The features of the spectrum used for this are the *g* values and the hyperfine splitting constants. The ESR spectrum of most nitroxyl spin adducts is dominated by the triplet splitting due to the nitrogen nucleus and, in the case of the nitrone compounds, by the supplementary splitting of the hydrogen atom attached in the beta position relative to the nitroxyl group. In the common case of PBN trapping a carbon-centered free radical, the adduct is formed in the beta position, and no significantly different spectral features are evident. Thus, various carbon-centered radicals trapped by, for example, PBN, will give rise to largely similar spectra with relatively minor differences in splitting constants and *g* values, and the unambiguous assignment of an ESR spectrum to a certain free radical is rather difficult. This problem can be eased in some cases by the use of ^{13}C-labeled substrates (*34*). If a nitroxide radical adduct is formed from a ^{13}C-centered free radical, the ^{13}C nucleus will influence the ESR spectrum and aid the unequivocal assignment of the spectrum. For example, if the ^{12}C-species gives a single-line spectrum, then the ^{13}C-analog will give a doublet; a mixture of the ^{12}C and the ^{13}C spin-trap adducts will thus produce a "triplet" where the strengths of the singlet versus the doublet will reflect the relative proportions of ^{12}C:^{13}C (*34*).

The spin-trapping technique has been applied by our group to demonstrate the production of free-radical intermediates during the metabolism of various xenobiotics.

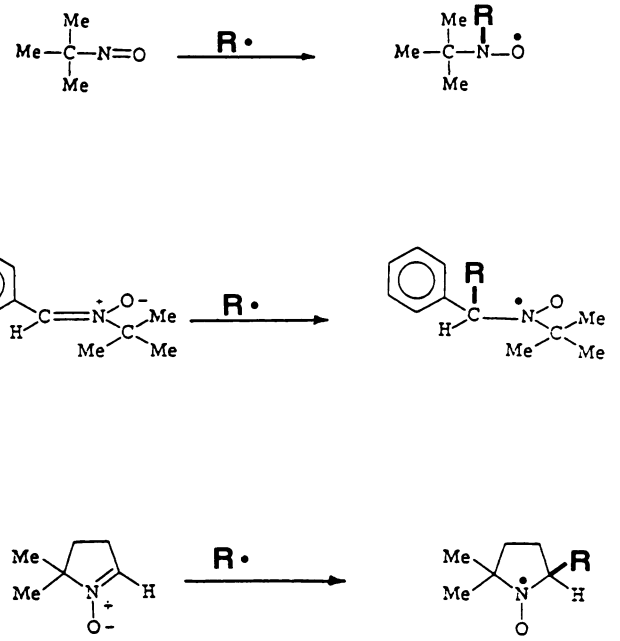

FIGURE 2. Spin adduct formation. The three most commonly used spin-trapping agents: MNP (top), PBN (middle) and DMPO (bottom). MNP adds free radicals in an alpha position to the nitroxide, while POBN and DMPO form the spin adduct beta to the nitroxide after addition to the C=N double bond. In the latter two cases, the ESR spectrum gives less information about the molecular structure.

Spin Trapping of Free Radicals Derived from CCl_4

The first attempts by this group to trap the $CCl_3^{\bullet}$ radical were not completely successful (*37*). In that study, CCl_4 was added to NADPH-supplemented microsomal suspensions containing the nitroso spin-trap MNP (Fig. 2). Although a nitroxide radical adduct was detected only when CCl_4 (or $CBrCl_3$) was present, and not with $CHCl_3$, it was evidently not derived directly from $CCl_3^{\bullet}$ and it was suggested that the species trapped was either $CCl_3O_2^{\bullet}$ or a secondary lipid peroxy radical.

In 1980, this group (*39*) and that of McCay (*40*) succeeded in trapping $CCl_3^{\bullet}$ with the nitrone spin trap PBN. The CCl_3–PBN spin adduct was found by both groups in rat liver microsomes incubated with CCl_4 and in the livers of rats dosed with CCl_4 and PBN *in vivo*. Tomasi et al. (*39*) also detected this product in isolated rat hepatocytes incubated with CCl_4.

The results were exciting and unambiguous: no ESR signal was obtained from hepatocytes or microsomes in the absence of CCl_4, a strong signal was obtained when CCl_4 was added and a clear supplementary splitting was apparent when ^{13}C-CCl_4 was used (Fig. 3). Successive papers by this group (*41,42*) further characterized various systems used for generating and trapping radicals from CCl_4. Physical techniques for free radical generation, such as UV- and γ-irradiation and pulse radiolysis were used in parallel to the biological systems in order to clarify the situation.

Irradiation experiments (*42*) gave the answer to the previously unexplained results (*34*) obtained using MNP in liver microsome suspensions. A mixture of MNP and CCl_4 was irradiated in the ESR spectrometer cavity with a 400 W lamp fitted with a filter to restrict the incident radiation to 300 to 360 nm, so avoiding the direct photolysis of MNP. In this way the spectrum shown in Figure 4 was obtained; this spectrum is characteristic of the CCl_3–MNP adduct, the features being due to the coupling of all three chlorine nuclei (see Table 1 for hyperfine splitting constants). The adduct is not stable, however, and decomposes either to nonradical products or, in the presence of oxygen, to a product (Fig. 4*b*) that almost certainly can be assigned to the ClCO–MNP adduct. The route of formation of this product is not clear, however. It seems therefore that the

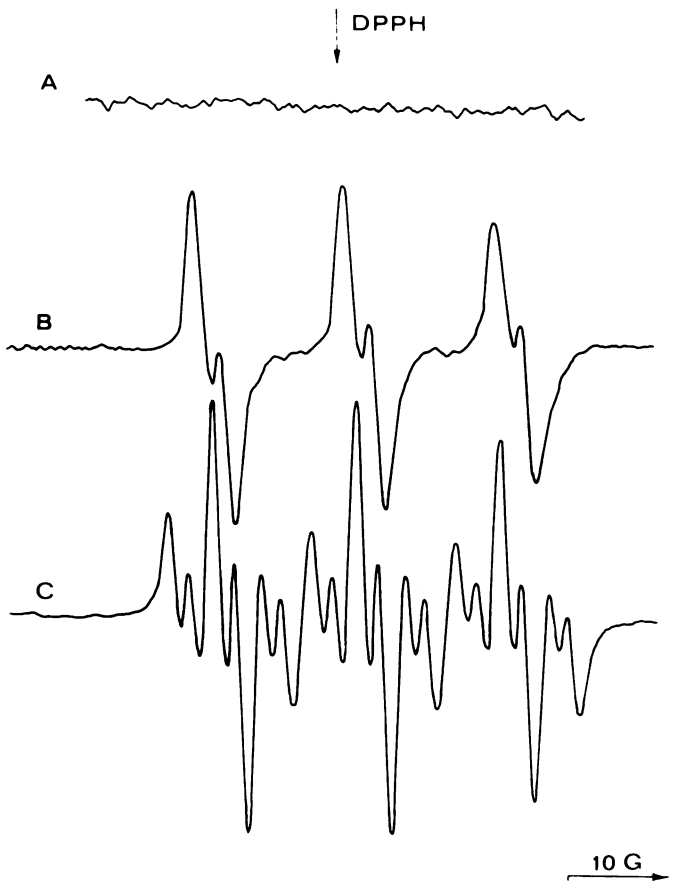

FIGURE 3. Example of a PBN spin adduct: (*A*) isolated rat hepatocytes incubated with PBN for 30 min showing no nitroxide spectrum; (*B*) as (*A*) but in the presence of CCl_4; (*C*) as above except using ^{13}C-CCl_4; the extra splitting due to the presence of ^{13}C nucleus is evident.

radicals trapped in the original microsomal experiments (*34*) were probably secondary lipid radicals arising from CCl_4-initiated lipid peroxidation.

PBN is clearly the superior spin trap for this particular task. Using irradiation techniques it was possible to investigate the effect of oxygen and measure, using competition methods (*43*) the rate constant for the reaction between the spin trap PBN and the free radicals

Table 1. ESR parameters of the various radical adducts detected by ESR spectrometry in the course of these investigations.

		Hyperfine coupling constants, G		
Radical	Spin trap	^{14}N	^{1}H	Other
$CCl_3^{\cdot}$	PBN	14	1.75	^{13}C, 9.68
$Cl^{\cdot}$	PBN	12.2	0.7	^{35}Cl, 6.1
$CCl_3O_2^{\cdot}$	PBN	13.5	1.6	
$CCl_3^{\cdot}$	MNP	13.1		^{35}Cl, 2.25
$ClCO^{\cdot}$	MNP	6.75		^{13}C, 5.7
$CHCl_2^{\cdot}$	PBN	14.7	2.37	^{13}C, 9.26
$CF_3CHCl^{\cdot}$	PBN	14.4	2.25	
$H_2CBrCH_2^{\cdot}$	PBN	14.5	2.15	^{13}C, 9.2

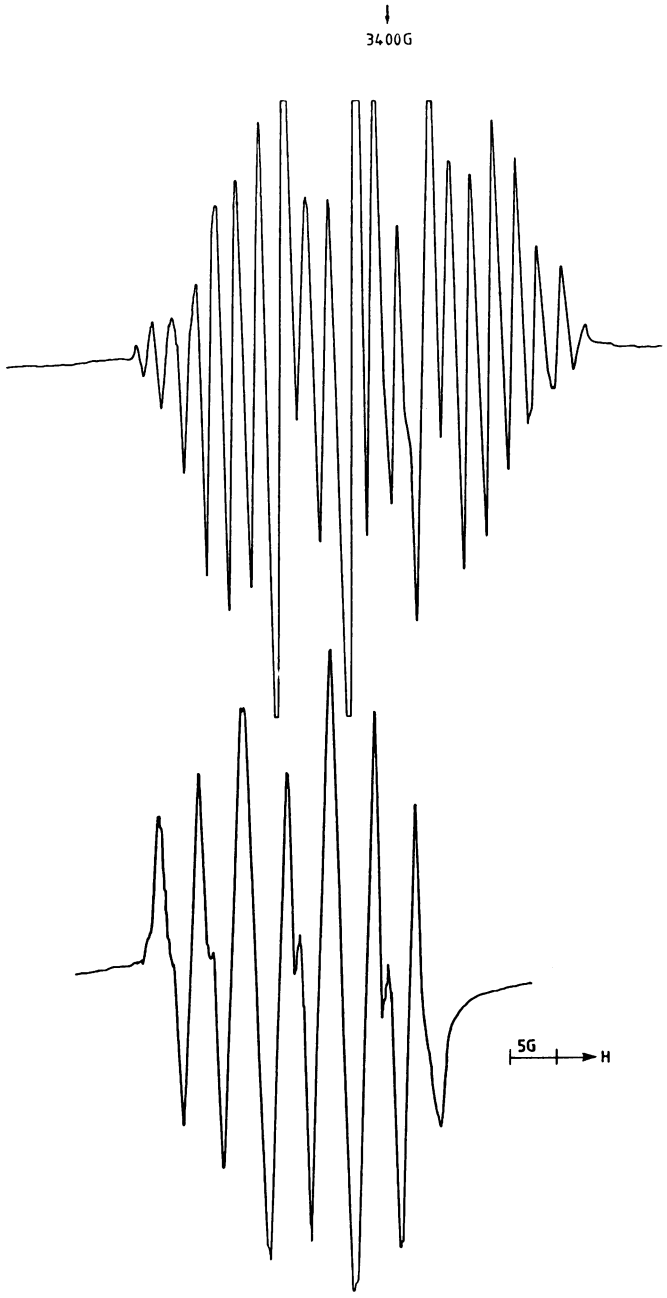

FIGURE 4. Example of MNP spin adducts: (*A*) ESR spectrum obtained after irradiating CCl_4 and MNP under hypoxic conditions. The lines are due to the coupling of the chlorine atoms to the nitroxide. (*B*) As above but in the presence of oxygen; the features have been assigned to the ClCO-MNP spin adduct.

$CCl_3^{\cdot}$ and $CCl_3O_2^{\cdot}$ formed in the absence and presence of oxygen, respectively (*39*). Only $CCl_3O_2^{\cdot}$ gave a measurable rate constant with PBN (5.4×10^6 $M^{-1}sec^{-1}$), the reaction of $CCl_3^{\cdot}$ being too slow for measurement in the pulse radiolysis system ($< 10^5$ $M^{-1}sec^{-1}$). This result was not unexpected, as the parallel studies of Packer, Willson and Slater in this department (see later)

had demonstrated the enhanced reactivity of the oxygenated radical species.

The γ-irradiation of CCl_4 at 77°K in the presence of PBN and the absence of oxygen enabled both $CCl_3^{\cdot}$ and $Cl^{\cdot}$ formed by homolysis of CCl_4 to be trapped (*42*). In the presence of oxygen and ^{13}C-CCl_4 the spectrum did not show the characteristic hyperfine features expected if the ^{13}C nucleus was attached beta to the nitrogen of PBN. The hyperfine splitting constants (Table 1) however, were slightly different from those of the $CCl_3^{\cdot}$ adduct, and it was proposed (*42*) that the spectrum be assigned to the CCl_3O_2–PBN adduct or, more likely, to the CCl_3O–PBN adduct resulting from molecular rearrangement of the CCl_3O_2–PBN species (*44,45*).

As expected, decreasing the concentration of oxygen in the incubation system increases the amount of $CCl_3^{\cdot}$ trapped by PBN, just as it increases the amount of covalent binding of radiolabeled CCl_4 to protein and lipid. In our hepatocyte system, hypoxic conditions were obtained by blowing humidified oxygen-free nitrogen over the surface of the suspension for 10 min; the remaining oxygen (ca. 50 μM) is rapidly consumed in less than 5 min of incubation, such that complete anoxia is obtained. Under these conditions, the intensity of the signal due to the CCl_3–PBN adduct is increased fivefold and covalent binding to cellular protein increases in parallel (Fig. 5). This further underlines the crucial role of the local oxygen concentration in determining the metabolic pathways subsequent to $CCl_3^{\cdot}$ formation. Spin-trapping experiments might give an indirect measurement of the relative importance of these pathways, but there are intrinsic difficulties in using this technique for quantitative measurements.

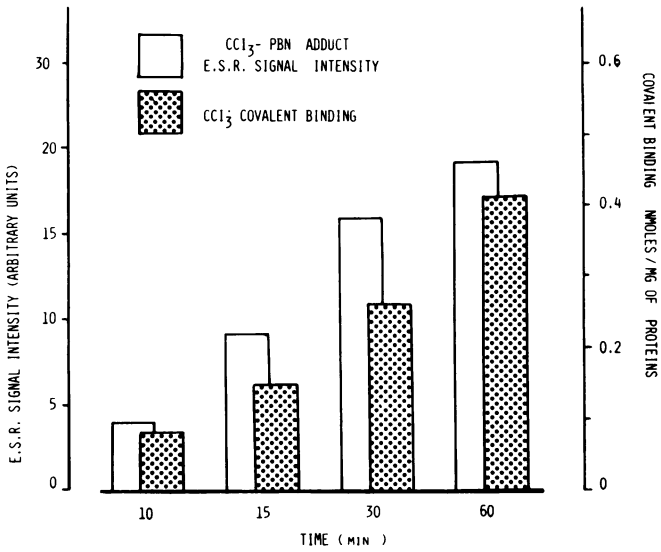

FIGURE 5. Covalent binding and spin trapping of $CCl_3^{\cdot}$. Parallel increase in the covalent binding of $CCl_3^{\cdot}$ to protein and the spin trapping of the same radical with PBN in isolated hepatocytes. Hepatocytes (7.5×10^6 cells/mL) were incubated in the presence of 0.15 mM ^{14}C-CCl_4 for the covalent binding experiments and with nonradioactive CCl_4 and 25 mM PBN for the spin-trapping experiments.

Spin Trapping of Radicals Derived from Other Haloalkanes

Halothane

Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is an anesthetic agent that produces rare and unpredictable liver damage (*46*). It can be metabolized by both aerobic and anaerobic pathways, but the latter route seems to be more important as regards hepatotoxicity. The one-electron reduction of halothane by the NADPH–cytochrome P-450 system to a free-radical product has been postulated, by analogy with the metabolic activation of CCl_4 (*46,47*). The C-Br bond is the weakest in the halothane molecule, and so the primary free radical product is likely to be the 1,1,1-trifluorochloroethyl radical ($CF_3C^{\cdot}HCl$) following bromide elimination. This is borne out by the finding that 1,1,1-trifluoro-2-chloroethane is a major metabolite of reductive halothane metabolism (*47*). Moreover, Trudell and co-workers have detected the 1,1,1-trifluoro-2-chloroethyl fragment bound to lipid following reductive halothane metabolism in reconstituted systems containing the enzymes of the NADPH-cytochrome P-450 system (*48,49*).

Using the experience gained from the aforementioned studies with CCl_4, we attempted to trap free-radical products of halothane metabolism in liver microsomes and in isolated hepatocytes; PBN was again used as the spin trap (*50*). When hepatocytes were isolated from phenobarbital-induced male rats and incubated in the presence of halothane and PBN under hypoxic conditions an ESR signal could be readily detected. The signal consisted of a triplet of doublets having nitrogen and hydrogen hyperfine splitting constants of 14.4 and 2.25 G, respectively (Table 1). Under aerobic conditions only a small unresolved spectrum was evident. If cells from noninduced male rats were used, no signal was found under either normoxic or hypoxic conditions. Isolated hepatocytes from phenobarbital-induced female rats gave rise to a similar ESR spectrum (but of a significantly lower intensity) when hypoxic conditions were used. Using liver microsomes from phenobarbital-induced male and female rats, the same ESR spectrum was found, again only under anaerobic conditions and once more higher in male-derived microsomes.

In all cases, the signal features were not sufficiently characteristic to permit an unambiguous identification. The spectrum seems unlikely, however, to be due to a PBN–lipid radical adduct, as it differs significantly from that reported by Kalynaraman et al. (*51*) for such a species. For the time being, we must assume that, based on the indirect evidence already mentioned, this spectrum is due to the trapping of the $CF_3C^{\cdot}HCl$ radical. These results differ from those of Poyer et al. (*52*), who reported a similar spin adduct but who did not find anaerobic conditions to be critical for its formation.

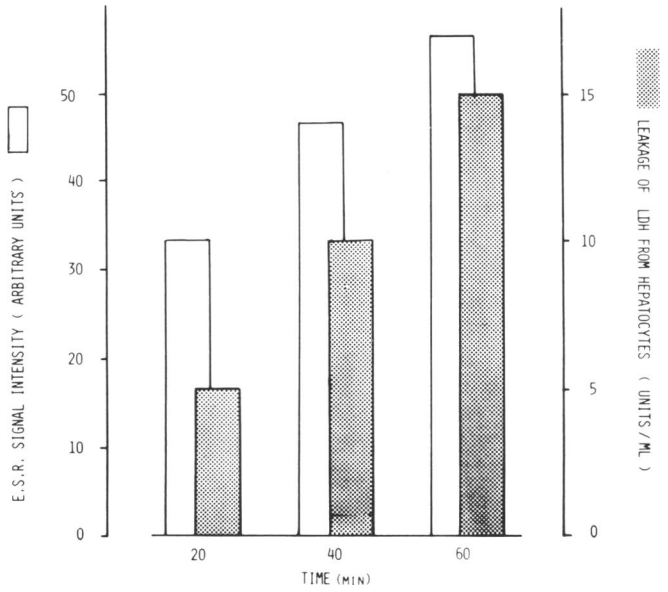

FIGURE 6. Time course of halothane radical-PBN spin adduct formation and LDH release. Hepatocytes were isolated from phenobarbital-induced rats and incubated anaerobically in the presence of 2 mM halothane. PBN was omitted from suspensions used for LDH assays; in the absence of halothane the leakage of LDH amounted to 8 units/mL after 60 min. The viability of the cells after 60 min incubation was 60% in the absence and 30% in the presence of halothane (*50*).

The relative intensity of the ESR signal obtained in liver preparations from male and female rats correlates well with the higher susceptibility shown by male rats, supporting the view that free radical intermediates might be involved in halothane hepatotoxicity. Also supportive of this postulate is the finding that radical adduct formation correlates directly with loss of cell viability in suspensions of isolated hepatocytes (Fig. 6). In the same experiments, halothane was found to induce lipid peroxidation in liver cells, albeit weakly, suggesting that this process may follow on from free radical formation and contribute towards cell damage. De Groot and Noll (*53*) have recently described the crucial role of oxygen concentration in halothane-induced lipid peroxidation.

These results do not, however, explain the rarity of halothane hepatotoxicity, and it seems increasingly likely that an immune response is involved, possibly resulting from changes in antigenic identity brought about by covalent binding of free radical intermediates.

Dibromoethane

1,2-Dibromoethane (DBE) is a chemical that has been used widely in industry and is still used as a fumigant in agriculture. It is remarkably toxic, producing both hepatic and renal damage, and is also carcinogenic (*54*). This molecule came under our scrutiny since its structure suggested the possibility that free radical intermediates might be produced during its metabolism. This possibility was studied in isolated hepatocytes and in liver microsomes (*55,56*).

Two main metabolic pathways have been reported: one involves a conjugation to glutathione (GSH) mediated by GSH transferase and eventually resulting in the formation of mercapturic acid derivatives, the other is based on oxidative dehalogenation by the microsomal drug metabolizing enzymes resulting in bromoacetaldehyde (*57,58*). The latter pathway is quantitatively the more important but it appears that the genotoxic effect is related to conjugation of DBE to GSH (*59*).

In our studies, incubation of DBE in suspensions of isolated hepatocytes in the presence of PBN did not give rise to any detectable spin adduct under normoxic conditions. If the oxygen tension was reduced, however, a well-resolved ESR spectrum was recorded (*55*) (Table 1). The identification of the radical so trapped was facilitated by the use of ^{13}C-labeled susbstrate. While this identified the radical as being derived from DBE under reductive conditions, there are still doubts about its precise structure; possibilities are $H_2CBrH_2C^{\bullet}$ or $H_3CHCBr^{\bullet}$, the latter deriving from the former by molecular rearrangement. The ^{13}C-labeled DBE certainly establishes that a radical intermediate is formed from DBE by interaction with the P-450 system, and that this has sufficient stability to be trapped by PBN. This is somewhat unexpected since it is known that radicals of the type $Hal\text{-}CH_2\text{-}C^{\bullet}H_2$ quickly break down to yield ethylenes.

This pathway to a radical intermediate may also be important *in vivo*, since conditions of low oxygen tension are also possible physiologically, especially in microenvironments of the liver (*60*). Spin trapping has thus enabled the discovery of a novel metabolic pathway for an important toxic compound. The contribution of this intermediate to the toxicity and carcinogenicity of DBE remains to be established but must be considered in any future investigations.

Chloroform

Chloroform ($CHCl_3$) is, as with all the trihalogenated methanes, hepatotoxic in both experimental animals and humans (*61*), although much less so than CCl_4; $CHCl_3$ is also carcinogenic in mice and rats (*62*). It is generally agreed that $CHCl_3$-induced liver damage is dependent upon its oxidative metabolism by the NADPH-cytochrome P-450 system to $COCl_2$ which then depletes cellular GSH and alkylates macromolecules.

In our experiments (*63*) with isolated hepatocytes, a radical product could be trapped under both hypoxic and normoxic conditions. The signal was some eightfold larger, however, under reduced oxygen tension. The use of $^{13}C\text{-}CHCl_3$ again enabled us to identify the radical as being $CHCl_3$-derived but it is not yet clear whether the radical trapped is the trichloromethyl or dichloromethyl species. As the hyperfine features (see Table 1) are different from those obtained using CCl_4 as substrate, the most likely candidate seemed to be $CHCl_2^{\bullet}$.

To prove this point, $CDCl_3$ was used since the higher stability of the C-D bond may be expected to produce a substantial isotope effect if cleavage of the C-H (or C-D) bond is involved in radical formation, i.e., if the radical species is $CCl_3^{\cdot}$. In fact, the use of $CDCl_3$ resulted in only a 20% decrease in signal intensity, not enough to rate as an isotope effect. No differences in the hyperfine splitting constants were apparent. Moreover, if cells were incubated with $CHBrCl_2$ an identical ESR spectrum was obtained but at least eight times more intense, reflecting the lower energy required to break the C-Br bond. Taken together these results suggest that the trapped radical product of $CHCl_3$ activation is $CHCl_2^{\cdot}$ and that, as in the case of halothane activation, the carbon-halogen bonds are preferentially broken.

Spin Trapping: Concluding Remarks

Apart from the examples shown above, we have studied the reductive metabolism of a range of halogenated methanes and ethanes to free radical intermediates in isolated hepatocytes under hypoxic conditions (*64*). Comparing the intensity of the ESR signal obtained from several halomethanes the tendency to form a free radical product appears to rank according to the number of halogens present in the molecule, thus:

$$CCl_4 > CHCl_3 > CH_2Cl_2$$

The electronegativity of the halogen atoms also directly influences the apparent ease of radical formation, for example:

$$CHI_3 > CHBr_3 > CHBrCl_2 > CHCl_3$$

or

$$CBrCl_3 > CCl_4 > CFCl_3$$

In the latter series, where $CCl_3^{\cdot}$ can be the common free radical product, the relative signal intensity of the PBN spin adducts is in good agreement with previous results on the ability to induce microsomal lipid peroxidation (*63*) and the hepatotoxicity of these halomethanes (*35*).

In summary, spin trapping is a powerful technique of great value to the identification of free radical intermediates in the metabolic activation of haloalkanes and, of course, other important xenobiotics. Some difficulties occur, however, concerning the identification of radical species so trapped and the quantitation of free radical formation. The value of ^{13}C-labeled and deuterated compounds have both been illustrated in the examples discussed above and have helped in removing some such difficulties. The use of HPLC techniques to purify spin adducts and mass spectrometry to definitively identify them will also be of great value in unravelling these remaining problems (*65,66*).

Measurement of the Reactivity of Radicals Derived from CCl_4

The general acceptance of the activation hypothesis in relation to the hepatotoxicity of CCl_4 brought with it the common assumption that the damaging effects on the liver resulted from the high reactivity of the primary free radical product, $CCl_3^{\cdot}$. However, until the mid-1970s no data were available on the precise reactivity of $CCl_3^{\cdot}$ under conditions comparable to those in living cells. The installation of a 4-MeV 200-nsec pulsed linear accelerator in the Biochemistry Department at Brunel University (*67*) led to the application of a new technique to the study of free radical involvement in the toxicity of CCl_4: pulse radiolysis.

Pulse radiolysis is a technique whereby a very short burst of high energy radiation, e.g., from a linear accelerator, is used to generate specific radical species in solution. The reactions of these radicals with other molecules can be monitored in the microsecond time scale by a variety of detection methods including spectrophotometry and conductivity measurements. This technique is fully reviewed by Willson (*68*).

$CCl_3^{\cdot}$ can be readily generated by dissociative electron capture in aqueous solutions containing excess amounts of scavengers that neutralize other reactive species (hydroxyl radicals, hydrogen atoms) formed during the radiolysis of water. Typically, aqueous solutions containing t-butanol or isopropanol and acetone are used; in the former case the principal reactions are:

$$H_2O \rightarrow OH^{\cdot}\ (45\%) + e^-_{aq}\ (45\%) + H^{\cdot}\ (10\%)$$

$$OH^{\cdot} + (CH_3)_3COH \rightarrow H_2O + (CH_3)_2COHCH_2^{\cdot}$$

$$e^-_{aq} + CCl_4 \rightarrow CCl_3^{\cdot} + Cl^-$$

By specifically generating $CCl_3^{\cdot}$ in this way, the reactions of CCl_4-derived radicals with various compounds of biological interest have been studied and the rate constants have been accurately measured.

In 1975, the results of the first of such investigations were published and they suggested that $CCl_3^{\cdot}$ was sufficiently electrophilic to react rapidly with various compounds including tryptophan, promethazine, and phenol (*69*). However, the authors pointed out that the solutions used were not rigorously deaerated and that the observed oxidations might therefore be due not to $CCl_3^{\cdot}$ but to its oxygenated form, the trichloromethylperoxy radical $CCl_3O_2^{\cdot}$. Subsequent investigations validated this point.

The measurements of reactivity with tryptophan, tyrosine, phenol, and promethazine were repeated but with stringent control of the oxygen concentration (*22*). In aerated solutions strong transient absorption spectra, similar to those first recorded, were observed in all cases. In deaerated solutions the absorptions were absent but re-introduction of air resulted in their reappearance. These observations showed that the $CCl_3^{\cdot}$ radical was not as reactive as had been previously

thought except that it reacted extremely rapidly with oxygen ($>10^9$ $M^{-1}sec^{-1}$). Moreover, the resultant $CCl_3O_2^{\cdot}$ radical was a very much more reactive species. In that paper, it was also suggested that this reaction might be the first step in the aerobic pathway of CCl_4 metabolism proceeding via phosgene to CO_2. It was also noted that this reaction explained the elevated level of covalent binding of CCl_4 metabolites in liver microsomes incubated anaerobically.

Subsequent investigations in this area were often carried out in close collaboration with Asmus and colleagues at the Hahn-Meitner Institute in Berlin. In fact, that group determined the precise rate constant for the reaction of $CCl_3^{\cdot}$ and oxygen to be $3.3 \times 10^9 M^{-1}sec^{-1}$, confirming that the reaction is practically diffusion-controlled (*70*).

Following the discovery of the high reactivity of $CCl_3O_2^{\cdot}$ in 1978, Packer, Willson, Slater and colleagues in Brunel University and Berlin investigated more deeply the reactions of this and related peroxy radicals with biologically important compounds. The $CCl_3O_2^{\cdot}$ radical was found to react extremely rapidly ($k = 5 \times 10^8$ M^{-1} sec^{-1}) with the biological antioxidant and vitamin, α-tocopherol (*71*). Similarly rapid reactions were also discovered with ascorbate ($k = 2 \times 10^8$ $M^{-1}sec^{-1}$) and with β-carotene ($k = 1.5 \times 10^9$ $M^{-1}sec^{-1}$) (*72*).

Packer has also confirmed the rapid reaction of $CCl_3O_2^{\cdot}$ with the amino acids tryptophan and tyrosine and also investigated the rates of reaction with the dipeptide tryptophanyl-tyrosine and with the enzyme lysozyme (*73*). In fact, Willson (*68*) has shown that $CCl_3O_2^{\cdot}$ will inactivate lysozyme more so than the $OH^{\cdot}$ radical that is actually a more powerful oxidizing agent. This apparent paradox arises since $CCl_3O_2^{\cdot}$ is sufficiently reactive to damage the enzyme but is more selective than $OH^{\cdot}$ in its targets and does not react with so many nonessential amino acid residues.

Another important aspect of the work of Packer and Willson and their colleagues is the study of the peroxy radicals derived from other halogenated aliphatic compounds including $CHCl_3$, CH_2Cl_2, CCl_3COOH, $CHCl_2COOH$, and $CClF_2COOH$ (*74*). The electrophilic reactivity of the peroxy radicals derived from these compounds, when tested against reactants including ascorbate, phenol, tyrosine, and promethazine, was found to increase with increasing substitution of the chlorine atoms. This finding was attributed to the inductive effect exerted by the halogen atoms. The electronegativity of the halogen atoms is therefore important in determining not only the ease with which haloalkanes will form free radicals but also in affecting their reactivity, or the reactivity of the peroxy derivatives. In view of the proposal that CCl_4-induced lipid peroxidation is due to the production of the $CCl_3^{\cdot}$ radical, it was clearly necessary to use the pulse radiolysis technique to measure the reactivity of $CCl_3^{\cdot}$ and $CCl_3O_2^{\cdot}$ with polyunsaturated fatty acids. Forni et al. (*75*) measured the rate of reaction of $CCl_3O_2^{\cdot}$ with oleic, linoleic, and arachidonic acids. The $CCl_3O_2^{\cdot}$ radical reacted rapidly with these fatty acids: the rate was dependent on their degree of unsaturation and ranged from 1.7×10^6 (oleic) to 7.3×10^6 $M^{-1}sec^{-1}$ (arachidonic). The reaction was proposed to proceed via hydrogen atom abstraction resulting in the formation of fatty acid radicals. Under anaerobic conditions the rates of reaction due to $CCl_3^{\cdot}$ were too slow for measurement by this technique ($<10^5$ $M^{-1}sec^{-1}$). These data promoted the revision of the prevailing hypothesis: it now seems likely that the major mechanism for the initiation of lipid peroxidation by CCl_4 involves $CCl_3O_2^{\cdot}$ rather than $CCl_3^{\cdot}$ (*76*).

Clearly, $CCl_3O_2^{\cdot}$ will react with a wide range of biological molecules at very rapid rates. It should be stressed that $CCl_3^{\cdot}$ is only relatively unreactive when compared to $CCl_3O_2^{\cdot}$. While reactions of $CCl_3^{\cdot}$ are too slow to be detected by the pulse radiolysis technique, they almost certainly do take place and surely contribute significantly to the overall metabolic perturbations that occur.

In summary, the realization that $CCl_3^{\cdot}$ reacts extremely rapidly with oxygen to form a highly reactive electrophilic species can be seen as a major advance, indeed, a watershed in the understanding of the damaging reactions induced by CCl_4 in living cells. Moreover, the formation of reactive peroxy radicals must be considered for other toxic haloalkanes.

Reactivity of Radicals Derived from Halothane

The pulse radiolysis investigations with CCl_4 led to similar studies with the anesthetic agent halothane (2-bromo-2-chloro, 1,1,1-trifluoroethane) that very occasionally produces an unexplained liver injury. It had been suggested that free radical intermediates might be involved in halothane toxicity (*77*). The predominant route for radical formation from halothane is assumed to be via bromide elimination:

$$CF_3CHBrCl \xrightarrow{e^-} CF_3CHCl^{\cdot} + Br^-$$

This halothane radical $CF_3{}^{\cdot}CHCl$ can be readily generated by pulse radiolysis and reacts rapidly with oxygen to form $CF_3CHClO_2^{\cdot}$. As in the case of CCl_4, the primary radical species was found to be much less reactive than the peroxy radical derivative (*78*). The latter species oxidized various substrates including ascorbate, α-tocopherol, propyl gallate, and several phenothiazines at rates in the range 10^8 to 10^9 $M^{-1}sec^{-1}$. No reaction was detectable with $CF_3CHCl^{\cdot}$, however ($k = <10^5$ $M^{-1}sec^{-1}$). The oxidizing power of the $CF_3CHClO_2^{\cdot}$, radical suggested it might be capable of initiating lipid peroxidation if it were formed in liver cell membranes. Forni et al. (*75*) measured the rate of reaction of $CF_3CHClO_2^{\cdot}$ with various fatty acids. As with $CCl_3O_2^{\cdot}$, this radical oxidized the fatty acids at rates proportional to their degree of unsaturation. In general, however, the rate constants for the reactions of $CF_3CHClO_2^{\cdot}$ with polyunsaturated fatty acids were about five times lower

than those for the $CCl_3O_2^{\cdot}$ radical. However, these data alone cannot predict the potential of halothane to stimulate lipid peroxidation: *in vitro*, CCl_4 is approximately 20 times more active than halothane in this respect (*63*).

As shown by De Groot and Noll (*79*), the oxygen tension is a critical factor in determining whether or not halothane will induce lipid peroxidation. The oxygen partial pressure must be low enough to allow the formation of halothane free radicals through the reductive pathway, but must be sufficiently high to permit lipid peroxidation.

Role of Cytochrome P-450 in the Activation of CCl_4 and Other Haloalkanes

CCl_4

As described earlier, it has been clear since the activation aspects of CCl_4 toxicity were first studied that the enzymes of the microsomal NADPH-cytochrome P-450 electron transport chain are responsible for CCl_4 metabolism. It is an unfortunate paradox that these enzymes, whose major function is to detoxify xenobiotics, also make certain compounds, such as CCl_4, more toxic.

The interaction of CCl_4 with the cytochrome P-450 system was studied in depth by Slater and Sawyer (*63,80,81*). In those studies, CCl_4-induced lipid peroxidation (measured by the thiobarbituric acid reaction) was taken as an index of CCl_4 metabolism. It was assumed that the effects of inhibitors on CCl_4-induced malonaldehyde (MDA) production represented underlying effects on CCl_4 activation; free radical scavengers that inhibited lipid peroxidation were postulated to scavenge $CCl_3^{\cdot}$. Our more recent work using other indices of CCl_4 metabolism, together with other data that have emerged since 1971, now shows that the situation is even more complex than was first thought. Unfortunately, all the practical indices of CCl_4 metabolism are indirect; detection of $CCl_3^{\cdot}$ directly by ESR spectrometry is not possible, and measurement of CCl_4 utilization is not sensitive. Instead, we have several indirect methods: CCl_4-induced lipid peroxidation, the covalent binding of radiolabeled CCl_4 to microsomal macromolecules, and the spin trapping of $CCl_3^{\cdot}$. Of these three, the measurement of covalent binding is probably the best, being more direct than the lipid peroxidation assay and more quantitative than spin trapping. Using all three techniques gives an insight into the pathways of CCl_4 metabolism.

Slater and Sawyer (*80*) compared CCl_4-induced MDA production with typical mixed-function oxidase (MFO) activity in terms of the effects produced by known inhibitors of the latter. Their conclusions were controversial, suggesting that the main locus of CCl_4 activation was not at cytochrome P-450 itself, but at some point proximal to it, e.g., "near to, if not identical with the NADPH flavoprotein." The results that pointed to this conclusion were based on selective inhibition studies and included: *p*-chloromercuribenzoate (pCMB) inhibited drug metabolism but stimulated CCl_4-induced MDA production; SKF 525A, at a concentration that inhibited aminopyrine demethylation, had no significant effect on CCl_4-induced lipid peroxidation, though a high concentration did inhibit it; CO, a classical P-450 inhibitor, actually stimulated CCl_4-induced MDA production.

These compounds, along with a wide range of other probe compounds, were re-investigated by our group in a series of experiments that added covalent binding and spin-trapping measurements to our methods of assessing CCl_4 metabolism (*41,82,83*), and the results are discussed below (Table 2).

The SH reagent pCMB is used at low concentrations to block electron flow to cytochrome P-450 and can inhibit the flavoprotein (*84*) and also cause denaturation of P-450 (*85*) at higher concentrations. It might be expected, therefore, that pCMB would inhibit normal drug metabolism and all parameters of CCl_4 metabolism. In fact, at 0.1 mM pCMB, CCl_4-induced MDA production is strongly stimulated, confirming the results of Slater and Sawyer (*80*); covalent binding of CCl_4 to protein is moderately inhibited, while aminopyrine demethylation is strongly reduced. At 0.2 mM pCMB, however, all tested parameters are strongly inhibited. Spin trapping of $CCl_3^{\cdot}$ is unaffected by 0.1 mM pCMB in microsomes but inhibited in hepatocytes. These results alone illustrate two important points: first, effects on CCl_4 metabolism should not be assessed using one parameter of this process, and second, using a single concentration of a test compound may give misleading results, as emphasized previously (*81*).

A similar dissociation between two parameters of CCl_4 metabolism is found with the surface active agent sodium dodecyl sulfate (SDS). With SDS a concentration-dependent inhibition of covalent binding is coupled with an increase in CCl_4-dependent lipid peroxidation (maximum at 1 mM), until the microsomes are completely solubilized when both activities are abruptly halted. Presumably, this reflects a gradual concentration-dependent breakdown of the membrane structure that uncouples the electron transport chain and also makes the membrane fatty acids more susceptible to peroxidative attack.

Menadione also can be considered as an agent that uncouples electron transport. With 100 μM menadione, all parameters of CCl_4 metabolism and MFO activity are strongly inhibited due to the diversion of electrons from NADPH:cytochrome P-450 reductase. Menadione cycles between its oxidized and semireduced forms, oxidizing NADPH and reducing oxygen to superoxide. At 1.0 μM, menadione is not an efficient electron-diverting agent, as evidenced by its small effect on MFO activity and on covalent binding of CCl_4. However, CCl_4-dependent MDA production is very profoundly reduced even by this low concentration. Cumene hydroperoxide-induced lipid peroxidation is not affected by this concentration (*83*). Together, these data suggest that it is

Table 2. Effects of inhibitors of cytochrome P-450-mediated drug metabolism on CCl_4-dependent lipid peroxidation, covalent binding of CCl_4 to protein, spin trapping of $CCl_3^{\cdot}$ and aminopyrine demethylation in rat liver microsomes.[a]

		% of control activity			
Agent[b]	mM	CCl_4-lipid peroxidation	^{14}C-CCl_4 binding	Spin trapping[c]	Aminopyrine demethylation
SKF 525A	0.02	—	—	—	46
	0.10	84	120	100 (74)	36
	0.50	38	195	—	—
Metyrapone	0.1	112	97	95 (96)	—
	1.0	105	33	—	28
	2.0	100	26		
CO		74	71	75 (80)	57
Pyrazole	5.0	95	103	—	35
Menadione	0.001	15	87	—	100
	0.1	0	14	20 (50)	15
pCMB	0.1	139	70	96 (75)	20
	0.2	28	18	—	0
SDS	1.0	140	57	—	—
	3.0	8	7	—	—
1,10-Ph	0.1	125	176	—	95
	2.2	207	403	—	—
2,2'-BP	5.0	105	172	—	41

[a] Data from the literature (*4,82,83,94*).
[b] Abbreviations: pCMB, *p*-chloromercuribenzoate; 1,10-Ph, 1,10-phenanthroline; 2,2'-BP, 2,2'-bipyridine.
[c] Values in parenthesis are those obtained in isolated hepatocytes.

the semiquinone form of menadione that is a powerful antioxidant or that menadione is scavenging a radical unique to the CCl_4-lipid peroxidation system, e.g., $CCl_3O_2^{\cdot}$.

SKF 525A, CO, and metyrapone are regarded as "classical" inhibitors of cytochrome P-450, and inhibition of a compound's microsomal metabolism by these agents is often taken as definitive proof that the compound is a substrate for cytochrome P-450. In our studies, and indeed in the hands of other workers, these inhibitors have not provided unequivocal evidence that CCl_4 is activated at cytochrome P-450.

In agreement with our earlier findings (*80,86*) CCl_4-induced production of MDA is found to be inhibited by SKF 525A if high concentrations are used in microsomal suspensions (*83*) and in hepatocytes (*87*); this effect requires a higher concentration than is needed to inhibit MFO activity, however. The spin-trapping of $CCl_3^{\cdot}$ in microsomes was not changed by 0.1 mM SKF 525A in microsomes, but some inhibition was seen in hepatocytes. The most striking effect of SKF 525A was to enhance strongly the covalent binding of CCl_4 to native microsomes. These results were obtained in the same samples as those where an inhibition of lipid peroxidation was found and illustrate again the apparent dissociation between these events. The enhancement of covalent binding found by us does not clarify the confused situation already present in the literature where reports of no effect (*88*), weak inhibition (*27*), and strong inhibition (*89*) are to be found. Interestingly, Sipes et al. reported a similar enhancement of binding of $CBrCl_3$ which is also activated to $CCl_3^{\cdot}$ (*88*). Also, metabolism of CCl_4 to $CHCl_3$ is reported to be stimulated by SKF 525 (*27*). As regards other pathways of CCl_4 metabolism, SKF 525A has been reported to inhibit weakly the conversion of CCl_4 to $COCl_2$ (*20*) and to electrophilic chlorine (*90*). Metabolism to CO_2 is reported to be inhibited strongly (*18*) or not at all (*19*). In none of these studies were the experimental conditions the same; variations in species, strain, and pretreatment of the animals and in the concentrations of oxygen and SKF 525A in the incubation medium preclude any meaningful comparisons. Moreover, in most of the studies just cited there were no dose response effects studied under similar conditions in relation to the inhibitory action of SKF 525A on the MFO system; in some of the studies only a single concentration of SKF 525A was used with the CCl_4 system, a procedure that is attended by risks to correct interpretation.

Inhibition with CO provides almost definite evidence for cytochrome P-450 involvement in a given microsomal metabolism reaction. In our studies the effects of CO on the microsomal metabolism of CCl_4 have not been sufficiently strong to be convincing. The small enhancement of CCl_4-induced microsomal lipid peroxidation reported by Slater and Sawyer (*80*) and reproduced in hepatocytes (*87*) was not confirmed in later experiments (*82*) where a weak inhibitory effect was generally found. Other workers also have been unable to demonstrate inhibition of this parameter by CO (*91,92*). Similarly, we have found covalent binding in microsomes and spin trapping of $CCl_3^{\cdot}$ in both microsomes and hepatocytes to be inhibited rather weakly by CO. However, other groups have found strong effects of CO on covalent binding of CCl_4 (*27,88*), on $CHCl_3$ production (*27,92*), on $COCl_2$ formation (*20*), and on conversion to electrophilic chlorine (*90*). Noguchi et al. (*93*) found that spin trapping of $CCl_3^{\cdot}$ was completely inhibited by CO in a reconstituted P-450 system but not in native microsomes. Again, the various different experimental conditions

used by these groups make direct comparison difficult but the inhibition of P-450-mediated reactions by CO is so fundamental that it should overcome these considerations. In the cases of $COCl_2$ and electrophilic chlorine, these products are again rather far removed from the activation event. The possibility that cytochrome P-450 is functioning to catalyze only later steps in this pathway must be considered. It is also worth bearing in mind the technical problems of ensuring that bubbling with CO sufficiently long enough to inhibit cytochrome P-450 does not eliminate significant amounts of CCl_4 or completely remove O_2 necessary for peroxidation events. In such experiments careful control of the CCl_4 and O_2 levels in the gassed suspension are therefore essential.

The third of the "classical" cytochrome P-450 inhibitors used here is metyrapone. No effects were observed on any of the indices of CCl_4 metabolism when metyrapone was added at 100 μM final concentration, a concentration that strongly depresses MFO activity. At 1–2 mM, covalent binding was strongly inhibited, in agreement with the result of Uehleke et al. (*27*), but CCl_4-dependent lipid peroxidation in the same experimental samples is not affected. Other type II ligands have also been tested (*82,83*) but no consistent effect is found. Covalent binding of CCl_4 in microsomes was inhibited by pyridine and its derivatives metyrapone and 2,3′-bipyridine, but not by imidazole, pyrazole, or 3-aminotriazole. CCl_4-dependent MDA production in microsomes was inhibited by pyridine, 2,3′-bipyridine, and 2,4′-bipyridine but not by metyrapone, imidazole, pyrazole, or 3-aminotriazole. All of these agents inhibited MFO activity.

Other pyridine analogs provided especially interesting data. These compounds, 2,2′-bipyridine and 1,10-phenanthroline, were originally tested with other metal-chelating agents in order to examine the role of iron in CCl_4-stimulated MDA production. It was surprising to find that 1,10-phenanthroline and, in some circumstances, 2,2′-bipyridine actually enhanced CCl_4-dependent lipid peroxidation. Further investigation revealed that the covalent binding of CCl_4 to microsomal protein was also enhanced; in fact, even more so (*94*). For example, 2.2 mM 1,10-phenanthroline doubled the rate of CCl_4-induced MDA production and increased the covalent binding of CCl_4 fourfold. This strong enhancing property is restricted among chelating agents to two compounds of similar structure suggesting that unspecific metal chelation *per se* is not involved. In fact, this effect is remarkably similar to the enhancement of microsomal aniline hydroxylation by these same compounds (*95*). However, in neither system is the mechanism of this unusual effect immediately apparent.

Considering these results with MFO-inhibitors together, several points can be made. It must be noted that various agents have differential effects on different parameters of CCl_4 metabolism. Hence, misleading results can be obtained if only one parameter is studied. Even taking covalent binding of CCl_4 as the best index of CCl_4 metabolism used here, the correlation with "typical" MFO activity is not good. However, divergent effects of certain inhibitors on different, verified MFO reactions are known. Moreover, CCl_4 metabolism is obviously not a typical MFO reaction, and it may therefore be inappropriate to expect it to respond in the same way to inhibitory compounds. Many of the results suggest that the pathways of CCl_4 metabolism leading either to covalent binding or lipid peroxidation are independent. One reason for this may be that $CCl_3^{\cdot}$ and $CCl_3O_2^{\cdot}$ are responsible for covalent binding and lipid peroxidation, respectively. It cannot be discounted that more than one locus of CCl_4 activation exists, but it may be that an artificial locus is created by the addition of certain compounds creating an artefactual electron flow (*76*).

The studies with specific MFO inhibitors have provided equivocal results concerning the precise locus of CCl_4 activation along the microsomal electron transport chain. However, other studies have provided reasonably strong evidence that cytochrome P-450 is the activation site.

Using $CoCl_2$ (*96*), allylisopropylacetamide (*88*), and cobalt protoporphyrin (*97*) to deplete cytochrome P-450 *in vivo* resulted in the decreased metabolism of CCl_4 *in vitro*. In the latter study, the use of cobalt protoporphyrin was inconclusive as NADPH:cytochrome P-450 reductase activity was also strongly reduced.

The use of reconstituted systems containing the MFO system enzymes has strongly implicated cytochrome P-450 as the site of CCl_4 activation; in such artificial systems these systems apparently did not metabolize CCl_4 in the absence of the hemoprotein (*23,90,92,93,98*). Interestingly, CCl_4 metabolism may be more rapid with certain isozymes of cytochrome P-450 than with others. Noguchi et al. (*93*) reported that the phenobarbitone-inducible form is the most active; Ingelman-Sundberg and colleagues (*99*) have reported the superior activity of the form induced by ethanol, benzene, or imidazole. Frank et al. (*100*) have found that most of the covalent binding of CCl_4 to microsomal protein is to cytochrome P-450 molecules. Various groups, including ourselves, have shown that such covalent binding may contribute directly to the destruction of this enzyme by CCl_4 (*101–103*).

If the role of cytochrome P-450 in CCl_4 activation now seems more acceptable the results obtained with so-called inhibitors of this enzyme require explanation, especially those showing differential effects on various indices of CCl_4 metabolism. Future studies will need to consider simultaneous measurement of all possible pathways of CCl_4 metabolism and must take into account the presence of certain cytochrome P-450 isozymes responding differently to CCl_4 and to the inhibitory compounds.

Other Haloalkanes

The involvement of our own group in investigating the role of cytochrome P-450 in the activation to free radicals of haloalkanes other than CCl_4 has so far been

Table 3. Effect of inhibitors of cytochrome P-450-mediated drug metabolism on the activation of various haloalkanes in isolated hepatocytes as measured by spin-trapping of their respective radical derivatives.[a]

Agent	% of control value			
	CCl_4	Halothane	DBE	$CHCl_3$
Menadione	50	—	—	—
pCMB	75	—	82	—
SKF 525A	74	100	76	81
Metyrapone	96	90	88	90
CO	80	10	0	5

[a] All compounds tested at a final concentration of 0.1mM (except for carbon monoxide for which there was a 60 sec exposure). Data from the literature (*41,55,64*).

limited to spin-trapping experiments in isolated hepatocytes. The other haloalkanes tested in this system are halothane, dibromoethane and chloroform (Table 3).

As with the case of CCl_4 so with halothane: the "classical" inhibitors of cytochrome P-450 did not provide unequivocal evidence for the role of this enzyme in reductive activation of halothane. SKF 525A and metyrapone failed to influence the formation of the halothane radical-derived ESR signal when used at 0.1 mM; high concentrations are not advisable in isolated hepatocytes as the cell viability is affected. At millimolar concentrations these compounds will inhibit anaerobic dehalogenation of halothane in subcellular fractions (*104*). On the other hand, CO strongly inhibited halothane radical formation, in contrast to its effects on CCl_4 metabolism. Indirect evidence for involvement of cytochrome P-450 is the greater activity in liver cells from male rats rather than females and the requirement for prior treatment with phenobarbitone.

In the case of dibromoethane (DBE) radical-adduct formation is inhibited by all three of the cytochrome P-450 inhibitors studied here. At 0.1 mM, SKF 525A and metyrapone are moderately effective (Table 3) and at 0.5 mM markedly so (*55*). CO is remarkably effective, almost completely suppressing the DBE radical-adduct signal, thereby demonstrating a very strong dependence on P-450, and emphasizing again the unusual nature of the corresponding results found with CCl_4.

Chloroform is metabolised aerobically to $COCl_2$, an oxygenase reaction that is known to be catalyzed by cytochrome P-450. The reductive pathway yielding the $CHCl_2^{\cdot}$ radical is also apparently dependent on this enzyme, as the CO again strongly reduced the radical-adduct signal. SKF 525A and metyrapone were again not sufficiently effective to be convincing. Probably the use of these two compounds at this level in isolated hepatocytes is unsuitable for the evaluation of the role of cytochrome P-450 in haloalkane metabolism.

Scavenging of Free Radicals Derived from CCl_4

The system of CCl_4-induced lipid peroxidation in rat liver microsomes was characterized in detail by Slater and Sawyer, and in that study the effects of several free radical scavengers were investigated (*81*). Promethazine and propyl gallate were especially effective inhibitors of this system and it was postulated that they act by scavenging the $CCl_3^{\cdot}$ radical that was presumed to be the initiator of lipid peroxidation. Our more recent investigations lead us to revise this hypothesis.

Promethazine, propyl gallate, catechin, and a range of other antioxidant compounds were tested in similar microsomal systems to those used originally (*63*) and the covalent binding of CCl_4 to microsomal protein was assayed simultaneously with CCl_4-dependent MDA production (*82,83*). Also, several such free-radical scavengers were tested for their effects on the spin-trapping of the $CCl_3^{\cdot}$ radical in microsomes and isolated hepatocytes (*41*).

In the covalent binding/lipid peroxidation experiments it was a general finding that CCl_4-induced lipid peroxidation was readily inhibited with free-radical scavengers, whereas the covalent binding of $CCl_3^{\cdot}$ to protein was not (Table 4). For example, 10 μM promethazine reduces CCl_4-induced MDA production to 13% of the control value whereas covalent binding remains at 84% of the control value. In the spin-trapping experiments, free-radical scavengers such as promethazine, propyl gallate, and catechin were all found to be ineffective in scavenging $CCl_3^{\cdot}$. Of course, in the latter experiments a competing scavenger, the spin trap itself, is present at much higher concentrations. These results are strongly suggestive that in inhibiting lipid peroxidation induced by CCl_4, these compounds are acting by scavenging radicals other than $CCl_3^{\cdot}$. They may be scavenging the propagating radicals of the peroxidation chain reaction. If they are also scavenging the initiating radical, then this radical is not identical to the intermediate that binds to protein.

Strong support is lent to this interpretation by the pulse radiolysis data on the reactivity of $CCl_3O_2^{\cdot}$, as discussed previously. Promethazine and other antioxidants react relatively slowly with $CCl_3^{\cdot}$ but very rapidly with $CCl_3O_2^{\cdot}$ which is produced from $CCl_3^{\cdot}$ at near diffusion-controlled rates in aerobic conditions (see earlier). Moreover, $CCl_3O_2^{\cdot}$ reacts more rapidly than does $CCl_3^{\cdot}$ with polyunsaturated fatty acids. It can be postulated, therefore, that the $CCl_3O_2^{\cdot}$ radical has the dominant role in inducing CCl_4-dependent lipid peroxidation. On the other hand $CCl_3^{\cdot}$ would be more likely to produce stable, covalently bound products. It should be emphasized that $CCl_3^{\cdot}$ may also play a minor role in inducing lipid peroxidation directly and that some covalently-bound products may be due to derivatives of the $CCl_3O_2^{\cdot}$ radical, e.g., $CCl_3O^{\cdot}$ or $COCl_2$ (see Fig. 1) and (*76*).

In inhibiting CCl_4-dependent lipid peroxidation, therefore, free-radical scavengers like promethazine may act in part by reacting with $CCl_3O_2^{\cdot}$, a reaction that is demonstrably rapid. That this is not their sole mechanism of action is indicated by their general antioxidant nature in other lipid peroxidation systems, suggesting that they scavenge lipid peroxy and alkoxy

Table 4. Effects of free-radical scavengers on CCl_4-induced lipid peroxidation, covalent binding of CCl_4 to protein, spin-trapping of $CCl_3^{\cdot}$ and aminopyrine demethylation on rat liver microsomes and (in parentheses) in rat hepatocytes.[a]

		% of control activity			
Agent	μM	CCl_4-lipid peroxidation	CCl_4 binding	$CCl_3^{\cdot}$ spin trapping	Aminopyrine demethylase
Promethazine	1.0	38	83	—	100
	10.0	13 (0)	84	—	79
	100.0	3	66	100 (86)	40
Propyl gallate	10.0	17 (42)	87	—	—
	20.0	13	88	—	—
	50.0	10 (21)	74	—	74
	100.0	—	—	100 (93)	—
Catechin	20.0	49	94	—	113
	50.0	30	94	—	—
	100.0	0 (0)	—	100 (100)	—

[a] Data from the literature (*41,81,83,87,109*).

radicals common to all such systems. In general, though, the CCl_4-dependent system is more susceptible to the inhibitory effects of these compounds (*105*). At high concentrations, the direct effects of these compounds on the electron transport chain must be considered, either competing for electrons at the reductase locus or binding to the substrate site at the terminal cytochrome. Propyl gallate, for example, inhibits drug metabolism at high concentrations (*106*) which probably explains the strong inhibition of the covalent binding of CCl_4 to microsomal protein obtained by Uehleke et al. (*27*) when using 1 mM of this scavenger. The inhibition of the microsomal metabolism of CCl_4 to CO_2 by promethazine (*18*) is of renewed interest, considering the probability that $CCl_3O_2^{\cdot}$ is probably an intermediate in this pathway.

The effects of the scavengers promethazine, propyl gallate, and catechin have been confirmed in hepatocytes: CCl_4-induced lipid peroxidation is inhibited, covalent binding of $CCl_3^{\cdot}$ to protein is not (*87,107*). This property of such scavengers can be used to probe the respective roles of covalent binding and lipid peroxidation in various aspects of cell damage caused by CCl_4. In this way we have shown that CCl_4-induced destruction of cytochrome P-450 in liver microsomes and in isolated hepatocytes is probably caused by a combination of direct binding of $CCl_3^{\cdot}$ to the hemoprotein and peroxidation of the surrounding membrane lipid (*102*). The CCl_4-induced inactivation of glucose-6-phosphatase, on the other hand, is due in most part to lipid peroxidation (*102*).

This approach was utilized also by Dianzani, Poli, and colleagues (*106*) using promethazine and propyl gallate to dissociate the effects of covalent binding from those of lipid peroxidation in CCl_4-induced blockage of lipoprotein secretion in isolated hepatocytes. It was concluded that covalent binding was the major contributor to this derangement.

While such experiments attempt to model the situation *in vivo*, the results may not be directly applicable: propyl gallate and promethazine are both effective against some aspects of CCl_4 hepatotoxicity, but catechin is not (*34,108,109*). In the whole animal there exists the problem of getting the scavenger to the "right place, at the right time and in the right concentration" (*110*). In addition, protective effects may be due not to scavenging action but to other effects on the organism. Promethazine, for example, when administered to rats, is found to have a synergistic effect with CCl_4 in increasing the breathing rate and decreasing the body temperature, and also delays the absorption of CCl_4 from the gastro-intestinal tract by approximately 2 hr (*111,112*). Thus, promethazine affords protection against some parameters of CCl_4 hepatotoxicity when measured 3 hr after dosing, partly because a smaller fraction of the CCl_4 dose has reached the liver at this time (*112*).

Concluding Remarks

In this short review we have summarized a number of the main contributions that have increased our knowledge of the metabolic activation of halogenated alkanes in general and CCl_4 in particular. There is no doubt in our minds that studies on the hepatotoxicity of CCl_4 have provided an unexpectedly, and probably uniquely large number of new concepts that relate to biochemical mechanisms of tissue injury. Nonetheless, many important problems concerning the toxicity of CCl_4 remain to be solved, particularly in the time band following metabolic activation, and when the network of cellular perturbations is expanding rapidly.

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